Preparation of *S*-Acetylthioglycoloyl Insulins Based on Separation by Anion-Exchange High-Performance Liquid Chromatography

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A method for the preparation of insulin derivatives having protected sulfhydryl group(s) on definite site(s) on the molecule which uses anion-exchange high performance liquid chromatography on a TSKgel DEAE-2SW column for separation is described. Porcine insulin reacts with *N*-succinimidyl *S*-acetylthioacetate to afford four species of insulin derivatives that have *S*-acetylthioglycoloyl group(s) at i) Gly(A1), ii) Gly(A1) and Phe(B1), iii) Gly(A1) and Lys(B29), and iv) Gly(A1), Phe(B1) and Lys(B29) positions. An insulin derivative which has a group at the Lys(B29)-position is prepared by the *S*-acetylthioglycoloylation of Gly(A1), Phe(B1)-dicitraconyl insulin followed by decitraconylation. The five derivatives are readily deacetylated with hydroxylamine to yield the corresponding sulfhydryl insulin derivatives.

Keywords *S*-acetylthioglycoloyl insulin; insulin; *N*-succinimidyl *S*-acetylthioacetate; cross-linking reagent; thiols preparation; high-performance liquid chromatography; anion-exchange chromatography

Insulin labeled with reactive group(s) at definite site(s) on the molecule should be useful for the preparation of site-specific insulin conjugates of enzymes and fluorescent substances, which may serve to improve sensitivity in enzyme- and fluoro-immunoassays of insulin and insulin antibodies.[1]

We previously reported two types of insulin derivatives, β-bromoacetamido-*n*-alkanoyl insulins[2] and 3-(2-pyridyldithio)propionoyl insulins,[3] which can react with sulfhydryl group(s) introduced onto a horseradish peroxidase molecule to give horseradish peroxidase–insulin conjugates.[2,3]

Insulin derivatives which have moiety(s) containing a sulfhydryl group at definite site(s) on the molecule should be promising alternative compounds that can be coupled with fluorophores and proteins substituted with halogenated moieties or those introduced with maleimide moieties. Sulfhydryl compounds are highly reactive but usually unstable in the air; the compounds should be protected with a group which is removable when the compound is required for use.

This paper described the preparation of *S*-acetylthioglycoloyl insulins (ATG-insulins; five species; Fig. 1), of which the acetyl group is eliminable under mild conditions in the presence of hydroxylamine. Porcine insulin, which has three amino groups at the Gly(A1), Phe(B1) and Lys(B29)-positions,[4] is reacted with *N*-succinimidyl *S*-acetylthioacetate (SATA)[5] to introduce *S*-acetylthioglycoloyl group(s) to the amino group(s). The reaction mixture is subjected to anion-exchange high-performance liquid chromatography (HPLC) to afford four species of ATG-insulins, Gly(A1)–ATG-, Gly(A1), Phe(B1)–diATG-, Gly(A1), Lys(B29)–diATG- and Gly(A1), Phe(B1), Lys(B29)–triATG-insulins. Lys(B29)–ATG-insulin is obtained by a reaction of SATA with Gly(Al), Phe(B1)-dicitraconyl insulin followed by decitraconylation.

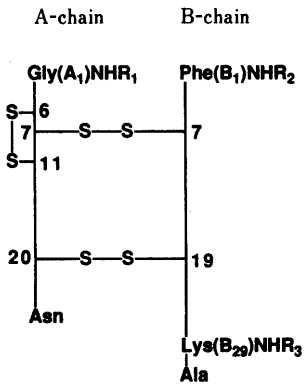

Fig. 1. Schematic Structures of ATG-Insulins

Gly(A1)–ATG-insulin: $R_1=CH_3COSCH_2CO$ (ATG), $R_2=R_3=H$; Gly(A1), Phe(B1)–diATG-insulin: $R_1=R_2=$ATG, $R_3=H$; Gly(A1), Lys(B29)–diATG-insulin: $R_1=R_3=$ATG, $R_2=H$; Gly(A1), Phe(B1), Lys(B29)–triATG-insulin: $R_1=R_2=R_3=$ATG; Lys(B29)–ATG-insulin: $R_1=R_2=H$, $R_3=$ATG.

Experimental

Reagents and Apparatus SATA was obtained from Sigma (St. Louis, Mo, U.S.A.). Lyophilized porcine insulin was prepared from a mono-component insulin solution (Insulin Novo Actrapid MC; Novo Ind., Copenhagen, Denmark) as previously described.[6] Other chemicals were of a reagent grade. Deionized water was passed through a Milli-QII system (Japan Millipore, Tokyo, Japan). Aqueous urea solution was deionized by mixing it with a mixed ion-exchange resin, Serdolit MB (particle size 0.3—0.8 mm; Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) prior to use, to eliminate the cyanate ion present as an impurity in urea. Molecular membrane tubes (Spectra/Por 3; molecular weight cutoff, approximately 3500; Spectral Medical Ind., Los Angeles, U.S.A.) were used for dialyses, which were normally carried out at 4 °C.

Absorbances were measured with a Hitachi 150-20 spectrophotometer using semimicro quartz cells (1 ml). Amino acid analyses were performed with a Hitachi 835 amino acid analyzer after hydrolysis of a protein sample in 6 M hydrochloric acid *in vacuo* at 110 °C for 24 h.

HPLC System and Its Operation A Hitachi 655 liquid chromatograph equipped with a Hitachi 650—60 recording processor, a Rheodyne 7125 syringe-loading sample injection valve (100-μl loop) and a Tosoh UV-8 model II spectromonitor operating at 280 nm was used. The column was TSKgel DEAE-2SW (300 × 7.8 mm i.d.; Tosoh, Tokyo, Japan) and the column temperature was ambient (20—25 °C). The mobile phase was 50 mM Na–K phosphate buffer (pH 7.0 or 6.0) containing 4 M urea, and the flow rate was 1.4 ml min^{-1}.

Procedure for the Investigation of Reaction Conditions To lyophilized porcine insulin (1.0 mg, 167 nmol) dissolved in 1.0 ml of 50 mM Na phosphate buffer (pH 6.0, 7.5, 8.0 or 9.0) was added 1—30-times molar excess of SATA dissolved in 50 μl of dried *N*,*N*′-dimethylformamide (DMF) at 0—50 °C with stirring. The mixture continued to be stirred at 0—50 °C for 0.5—60 min. A portion (100 μl) of the resulting mixture was subjected to HPLC using 50 mM Na–K phosphate buffer (pH 7.0) containing 4 M urea as a mobile phase.

Preparation of Four ATG-Insulins To lyophilized porcine insulin (20.0 mg, 3.33 μmol) dissolved in 20 ml of 0.1 M Na phosphate buffer (pH 9.0) was added five portions (22 μl) of 150 mM SATA (total 16.5 μmol) in dried DMF at intervals of 1 min during 4 min at 25 °C with vigorous stirring, and the mixture continued to be stirred for another 6 min. The

resulting mixture was treated by chromatography on a Sephadex G-25 (200 g; Pharmacia LKB Biotechnology, Uppsala, Sweden) column (360 × 60 mm i.d.) using 1 mM Na phosphate buffer (pH 7.0) to remove any SATA which remained unreacted. The protein fraction was collected and lyophilized to give a mixture of ATG-insulins and intact insulin (19 mg). The mixture was dissolved in 1.0 ml of 50 mM Na–K phosphate buffer (pH 7.0) containing 4 M urea. Portions (100 μl) of the solution were subjected to HPLC using 50 mM Na–K phosphate buffer (pH 6.0) containing 4 M urea. The combined individual eluates (for fractions see Fig. 5) were dialyzed against 2 l of 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA·2Na) (4 times) and 2 l of water and lyophilized. The lyophilizates were dissolved in 4.0 ml of 0.1 M Na phosphate buffer (pH 9.0), respectively, and subjected to chromatography on a Sephadex G-25 (5.6 g) column (360 × 10 mm i.d.) with 1 mM Na phosphate buffer (pH 7.0) to remove any remaining urea and EDTA. The respective protein fractions were dialyzed against 1 l of water (5 times) and lyophilized to give the following ATG-insulins (all colorless powder) [yield (mg), UV λ_{max} (nm) (in 0.1 M Na phosphate buffer (pH 9.0)) and ε, in that order, in parentheses]: Gly(A1)–ATG-insulin (2.74, 275.5, 7.59×10^3); Gly(A1), Phe(B1)–diATG-insulin (1.74, 275.5, 7.69×10^3); Gly(A1), Lys(B29)–diATG-insulin (2.12, 275.5, 7.82×10^3); Gly(A1), Phe(B1), Lys(B29)–triATG-insulin (1.50, 275.0, 8.10×10^3). The position of *S*-acetylthioglycoloyl groups of the ATG-insulins was determined by the method of Levy,[7] with minor modifications as previously described.[3]

Preparation of Lys(B29)–ATG-Insulin A portion (400 μl) of 150 mM SATA solution in dried DMF was added to a solution of Gly(A1), Phe(B1)-dicitraconylinsulin[6] (12 mg, 2.0 μmol) in 12 ml of 0.1 M Na phosphate buffer (pH 9.0) at 25 °C with vigorous stirring, and the mixture was stirred at 25 °C for 10 min. The resulting mixture that contained the yielded Gly(A1), Phe(B1)–dicitraconyl-Lys(B29)–ATG-insulin was dialyzed against 1 l of 0.1 M acetic acid (pH 2.9) (5 times) at 25 °C for 24 h to decitraconylate the compound, then lyophilized. The residue was dissolved in 500 μl of 50 mM Na–K phosphate buffer (pH 7.0) containing 4 M urea. Portions (100 μl) of the solution were subjected to HPLC using the phosphate buffer. The combined protein fraction was dialyzed against 2 l of 1 mM EDTA·2Na (4 times), then against 2 l of water and lyophilized. The residue was dissolved in 1.0 ml of 0.1 M Na phosphate buffer (pH 9.0) and treated by a Sephadex G-25 (5.6 g) column (360 × 10 mm i.d.) chromatography using 1 mM Na phosphate buffer (pH 7.0) to eliminate EDTA and any urea which remained unremoved. The protein fraction was collected and dialyzed against 1 l of water (5 times). The lyophilization of the fraction left Lys(B29)–ATG-insulin [colorless powder, yield 3.11 mg; UV λ_{max} (nm) (in 0.1 M Na phosphate buffer (pH 9.0) and ε, 275 and 7.39×10^3, respectively]. The position of the *S*-acetylthioglycoloyl group was confirmed by the above-mentioned method.

Deacetylation of ATG-Insulins and Determination of a Sulfhydryl Group in Deacetylated ATG-Insulins To an ATG-insulin (333 nmol) solution in 1.0 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M sodium chloride and 5 mM EDTA·2Na (evacuated) was added 500 μl of 1.0 M hydroxylamine hydrochloride solution in the buffer, and the mixture was stirred at 30 °C for 20 min. The resulting mixture was dialyzed against 1 l of 0.1 M Na phosphate buffer (pH 6.0) containing 50 mM sodium chloride and 5 mM EDTA·2Na (evacuated) (4 times). Approximately 1.5 ml of a thioglycoloyl insulin solution (220 nmol ml^{-1}) was obtained. The sulfhydryl group in the insulins was determined by the 4,4′-dithiodipyridine method.[8]

Results and Discussion

The reaction mixture of insulin with SATA could be successfully separated by anion-exchange HPLC on a DEAE-2SW column using a neutral (pH 7.0) or weakly acidic (pH 6.0) phosphate buffer containing urea at a relatively high concentration (4 M); in the absence of urea, the components of the mixture were intensely retained on the column. Also, the retention was increased with a phosphate buffer at pHs lower than 6, but was decreased at pHs higher than 7. Figure 2 depicts a chromatogram of a reaction mixture obtained by treating insulin with a 5-times molar excess of SATA at pH 9 at 25 °C for 10 min according to the procedure for the investigation of reaction conditions. The peaks for Gly(A1)–ATG-, Gly(A1), Phe(B1)–diATG-, Gly(A1), Lys(B29)–diATG- and Gly(A1), Phe(B1), Lys(B29)–triATG-insulins were detected in the chromatogram.

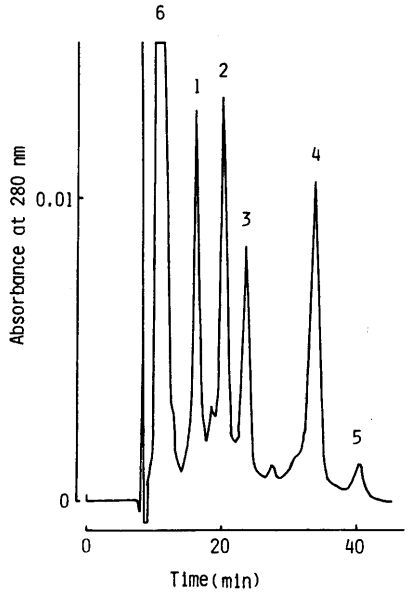

Fig. 2. Chromatogram Obtained with a Reaction Mixture of Insulin with SATA

Insulin (1.0 mg) was treated (for procedure details see text) and the mixture was chromatographed using 50 mM Na–K phosphate buffer (pH 7.0) containing 4 M urea. Peaks: 1, insulin; 2, Gly(A1)–ATG-insulin; 3, Gly(A1), Phe(B1)–diATG-insulin; 4, Gly(A1), Lys(B29)–diATG-insulin; 5, Gly(A1), Phe(B1), Lys(B29)–triATG-insulin; 6, SATA; others, unidentified.

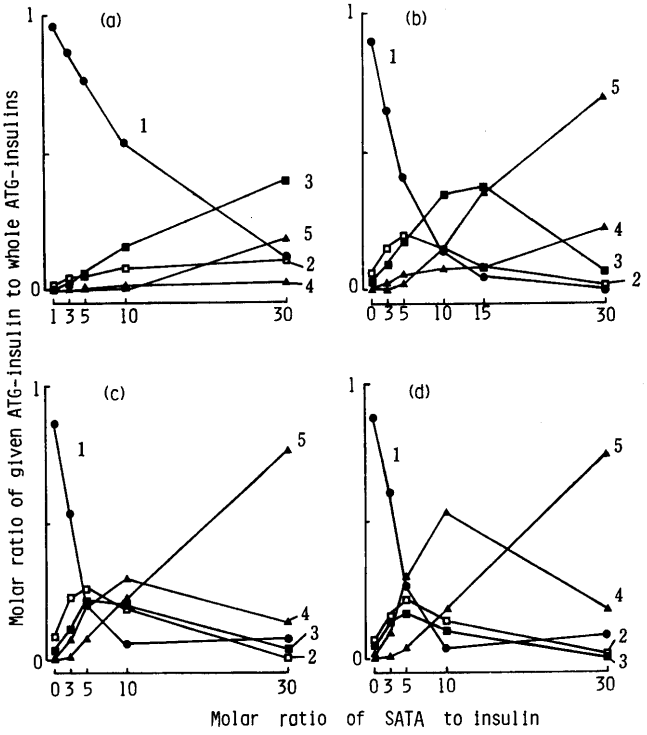

Fig. 3. Effect of the Molar Ratio of SATA to Insulin and pH on the Formation of ATG-Insulins

Insulin (1.0 mg) was treated according to the procedure at the molar ratios of SATA to insulin of 1—30, at 25 °C at pHs (a) 6.0, (b) 7.5, (c) 8.0 and (d) 9.0 for 10 min. Curves: 1, insulin; 2, Gly(A1)–ATG-insulin; 3, Gly(A1), Phe(B1)–diATG-insulin; 4, Gly(A1), Lys(B29)–diATG-insulin; 5, Gly(A1), Phe(B1), Lys (B29)–triATG-insulin.

The peaks in the chromatogram were identified by the coincidence of their retention times with those of

the authentic ATG-insulins prepared in the experimental section and an increase in peak heights in the co-chromatography with the authentic samples. Peaks for possible ATG-insulins, Lys(B29)– and Phe(B1)–ATG-insulins and Phe(B1), Lys(B29)–diATG-insulin, were not found.

The molar ratio of SATA to insulin and pH in the reaction affected the formation of the four ATG-insulins (Fig. 3). In the reaction at 25 °C for 10 min, the yield of Gly(A1), Phe(B1), Lys(B29)–triATG-insulin increased with increasing molar ratio and pH, and Gly(A1), Phe(B1)– and Gly(A1), Lys(B29)–diATG-insulins were produced more readily at pHs of 6.0—7.5 and 8.0—9.0, respectively; the reaction at the molar ratio of 5 at pH 9.0 provided almost maximum production of the four ATG-insulins.

Under these conditions, the formation of Gly(Al)–ATG- and Gly(Al), Phe(B1)–diATG-insulins decreased with a rise in reaction temperature in the range 0—37 °C, and that of Gly(A1), Lys(B29)–diATG- and Gly(A1), Phe(B1), Lys(B29)–triATG-insulins was maximum at 10 and 25 °C, respectively (Fig. 4); the temperature of 25 °C, which was selected in the procedure, could afford the four ATG-insulins in fairly high yields, respectively. The reaction time (0.5—60 min) did not greatly affect the formation of the ATG-insulins; the reaction for 10 min was employed.

The reaction of insulin with SATA was thus conducted in a preparative scale (using 20 mg of insulin) under the above-mentioned conditions. Figure 5 shows a chromatogram of the reaction mixture. The eluates from the shaded peaks were collected to give the four ATG-insulins in relatively good yields. Unexpectedly, a peak corresponding to Lys(B29)–ATG-insulin (peak 3 in Fig. 5) was found in the chromatogram. The peak partly overlapped the peak for Gly(A1)–ATG-insulin (peak 2) when a phosphate buffer of pH 7.0 added with urea was used as a mobile phase. Although the separation of peak 3 from peak 2 was much improved by using a phosphate buffer of pH 6.0 containing urea, the separation was still unsatisfactory for the isolation of Lys(B29)–ATG-insulin; this was separately prepared through Gly(A1), Phe(B1)–dicitraconyl-insulin as described in the experimental section.

The position of *S*-acetylthioglycoloyl group(s) in the ATG-insulins was determined by deaminating the insulins and by measuring the number of glycyl, phenylalanyl and lysyl residues according to a modified Levy method[3] (Table I); there is no discrepancy between the theoretical values of the amino acid residues and those found.

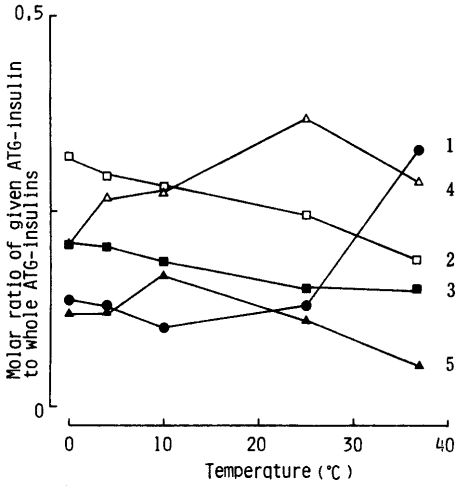

Fig. 4. Effect of the Reaction Temperature on the Production of ATG-Insulins

Insulin (1.0 mg) was treated as in Fig. 3 but at the molar ratio of SATA to insulin of 5 at pH 9.0 at various temperatures. For curves see Fig. 3.

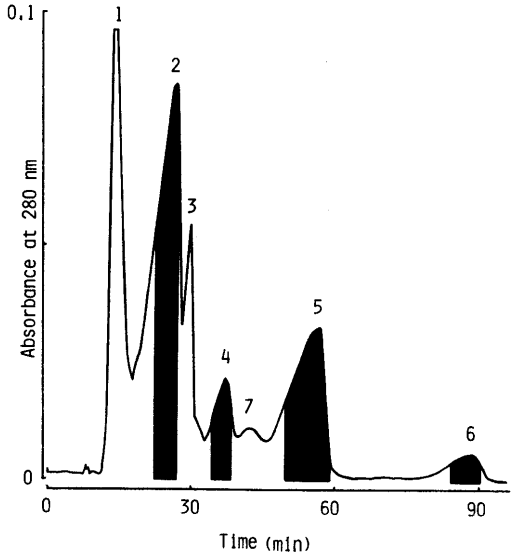

Fig. 5. Chromatogram Obtained in the Preparative Separation of ATG-Insulins

Insulin (20.0 mg) was treated by the procedure for the preparation of four ATG-insulins, and the eluates of the shaded parts of peaks were collected. Peaks: 1, insulin; 2, Gly(A1)–ATG-insulin; 3, Lys(B29)–ATG-insulin; 4, Gly(A1), Phe(B1)–diATG-insulin; 5, Gly(A1), Lys(B29)–diATG-insulin; 6, Gly(A1), Phe(B1), Lys(B29)–triATG-insulin; 7, unidentified.

TABLE I. Number of Glycyl, Phenylalanyl and Lysyl Residues of the ATG-Insulins after Deamination

Position of *S*-acetyl-thioglycoloyl group	Number of amino acid residues per molecule[a]					
	Gly		Phe		Lys	
	Theoretical	Found	Theoretical	Found	Theoretical	Found
Procine insulin	3	2.71	2	1.85	0	0.01
Gly(A1)	4	3.95	2	1.72	0	0.00
Lys(B29)	3	3.05	2	2.04	1	1.02
Gly(A1), Phe(B1)	4	4.16	3	3.03	0	0.08
Gly(A1), Lys(B29)	4	3.80	2	2.05	1	0.97
Gly(A1), Phe(B1), Lys(B29)	4	4.09	3	3.01	1	0.95
Intact porcine insulin[b]	4	3.89	3	2.99	1	0.97

a) Calculated based on six leucyl residues per porcine insulin molecule. *b*) Not subjected to deamination.

TABLE II. Number of Sulfhydryl Groups of the ATG-Insulins after Deacetylation

Position of *S*-acetyl-thioglycoloyl group	Number of sulfhydryl groups per molecule[a]	
	Theoretical	Found
Porcine insulin	0	0.00
Gly(A1)	1	1.00
Lys(B29)	1	0.93
Gly(A1), Phe(B1)	2	1.78
Gly(A1), Lys(B29)	2	1.83
Gly(A1), Phe(B1), Lys(B29)	3	2.83

a) Determined by the 4,4'-dithiodipyridine method.[8]

A perfect deacetylation of the ATG-insulins could be achieved under mild conditions using hydroxylamine, without reduction of the disulfide bonds on the insulin molecule (Table II).

In conclusion, this paper provided the first method for the preparation of insulin derivatives that have protected sulfhydryl group(s) at definite site(s) on the molecule, and the ATG-insulins should be useful for the preparation of insulin conjugates with informative substances such as enzymes and fluorophores.

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References

1) K. Zaitsu, M. Nakayama, M. Nanami and Y. Ohkura, *Chem. Pharm. Bull.*, **39**, 499 (1991).
2) K. Zaitsu, M. Ohnishi, H. Hosoya, H. Sugimoto and Y. Ohkura, *Chem. Pharm. Bull.*, **35**, 1991 (1987).
3) K. Zaitsu, M. Nakayama and Y. Ohkura, *Chem. Pharm. Bull.*, **36**, 1425 (1988).
4) T. Blundell, G. Dodson, D. Hodgkin and D. Mercola, "Advances in Protein Chemistry," Vol. 26, ed. by C. B. Anfinsen, Jr., J. T. Edsall and F. M. Richards, Academic Press, New York and London, 1972, pp. 279—402.
5) R. Julian, S. Duncan, P. D. Weston and R. Wrigglesworth, *Anal. Biochem.*, **132**, 68 (1983).
6) K. Zaitsu, H. Hosoya, Y. Hayashi, H. Yamada and Y. Ohkura, *Chem. Pharm. Bull.*, **33**, 1159 (1985).
7) D. Levy, *Biochim. Biophys. Acta*, **317**, 473 (1973).
8) D. R. Grassetti and J. F. Murray, Jr., *Arch. Biochem. Biophys.*, **119**, 41 (1967).